

### REMARKS

Claims 1-11, 21-30, 63, 64 and 69 were subject to examination in the Final Action dated November 26, 2004. Applicants note with appreciation that the Examiner has indicated that the subject matter of claims 2, 4, 10, 11, 22, 27-29, 52 and 64 is free of the cited art and are only objected to for being dependent from rejected claims. Claim 3 has been amended herein, and withdrawn claims 8, 9, 12-20, 25, 26, 31, 33-43, 46-51, 53, 55-62 and 65-68 have been canceled. The remaining issues relate to three obviousness rejections over Ling et al., Sukhwinder et al., Barker et al., Barcaccia et al., Dice and/or Tulloss, which will be addressed below in the order raised in the Final Action.

**I. Claims 1, 3, 5-7, 21, 23-24, 30, 63 and 69 are Patentable over Ling et al. in view of Barcaccia et al. as Defined by Dice.**

A new rejection has been issued on the basis that claims 1, 3, 5-7, 21, 23-24, 30, 63 and 69 are allegedly unpatentable under §103(a) for obviousness over Ling et al. in view of the newly cited reference Barcaccia et al. (*J. Horticultural Science and Biotechnology* 74:243-50, 1999) as further defined by Dice. The Final Action states that "Ling et al. teaches a method of distinguishing genetic relationship and diversity between Poinsettia cultivars, including breeding family 'Freedom'." The Final Action also states that "Barcaccia et al. teach a method of using an AFLP marker protocol to distinguish genetic relationships and diversity of *Pelargonium peltatum*, an ornamental asexual plant" and further "Barcaccia et al. teach that AFLP fingerprinting combines the reliability of the RFLP assay with the efficiency of PCR technique and AFLP markers provided a much more powerful and reliable tool." The Examiner concludes that it would have been obvious to "improve the method of identifying poinsettia cultivars by RAPD markers as taught by Ling et al. to include the AFLP marker assay as taught by Barcaccia et al. because Barcaccia et al. teaches of the advantages of using the AFLP procedure to analyze genetic relationships and diversity in ornamental plants." Applicants respectfully disagree with this rejection.

As Applicants have previously addressed at some length, the Ling et al. reference does not disclose or suggest a method of estimating a genetic relationship

between poinsettia plants, a method of determining the profile similarity between a poinsettia plant and a known poinsettia cultivar, a method of estimating a genetic relationship of a first poinsettia plant to a poinsettia plant that is a representative member of a specific breeding family, a method of assessing the breeding history of a poinsettia plant, a method of determining whether a poinsettia plant is a representative of a known poinsettia cultivar, or a method of distinguishing a poinsettia cultivar from a known poinsettia cultivar using AFLP analysis as recited by the present claims. As conceded by the outstanding rejection, Ling et al. concerns RAPD analysis.

The outstanding rejection is based on the premise that Barcaccia et al. provides the motivation for one of ordinary skill in the art to apply AFLP analysis to poinsettia because Barcaccia et al. used this technique to evaluate *Pelargonium* (geranium) cultivars and stated AFLPs were superior to RAPDs. However, there is absolutely no suggestion in the cited Barcaccia et al. publication that AFLP analysis can be applied to poinsettia or even a more general statement that AFLP analysis would be suitable for the study of ornamental plants other than geranium. Barcaccia et al. is solely concerned with geraniums and the applicability of AFLPs to geranium cultivars. As described in the enclosed Declaration of James W. Moyer under 37 C.F.R. §1.132 (*hereinafter*, "the Moyer Declaration"), Barcaccia et al.'s work in geranium is not predictive of the outcome in poinsettia (Moyer Declaration, para. 5).

Thus, as a preliminary matter, there is no objective basis for the combination of Ling et al. and Barcaccia et al. and, further, even if the two references are combined, they would not have provided motivation to one of ordinary skill in the art at the time of invention to practice the presently claimed methods.

Prior to the studies described in the present application, it would not have been at all obvious that AFLP fingerprinting analysis could be successfully applied to poinsettia (Moyer Declaration, para. 5). There were some reports of AFLP analysis in other ornamental plants, but it was uncertain from these studies whether there would be sufficient inter-cultivar diversity among poinsettias that would be detectable by AFLPs (Moyer Declaration, para. 5). As already discussed, poinsettias are asexually reproducing, and most cultivars have been identified by selection of sports

or mutation breeding. As a result, the genetic base of poinsettias is very narrow (Moyer Declaration, para. 6). One of ordinary skill in the art could not have had any reasonable expectation of success prior to the present invention that sufficient polymorphisms detectable by AFLP would exist among poinsettia cultivars (Moyer Declaration, para. 6).

The Court of Appeals for the Federal Circuit has held that "[b]oth the suggestion and the expectation of success must be founded in the prior art, not in the applicant's disclosure." *In re Dow Chemical*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). These criteria are not satisfied by the outstanding obviousness rejection.

At most, the combination of the Ling et al., Barcaccia et al. and Dice references would have made it obvious to try to apply AFLPs to poinsettia cultivars. However, in the absence of any suggestion or demonstration whatsoever in any of the cited references that AFLP analysis would be appropriate for the study of poinsettias, there could have been no reasonable expectation of success with respect to the present invention. As "obvious to try" is a legally insufficient basis for rejection under §103(a), the outstanding rejection should be withdrawn.

Further evidence of the nonobviousness of the present invention comes from the inventors' work with microsatellite analysis of poinsettias (described in paragraphs 8-15 of the Moyer Declaration). Although microsatellites are generally considered more powerful than AFLPs (Moyer Declaration, para. 13), the study described in the Moyer Declaration demonstrates that microsatellites were not useful for genetic analysis of poinsettias. This failure appears to be due to a lack of sufficient polymorphisms at the SSR loci of poinsettias which, in turn, is likely attributable to the narrow genetic base of poinsettias (Moyer Declaration, para. 13).

Each of the fingerprinting methods discussed above, RAPD, AFLP and microsatellites detect different markers within the plant genome. The microsatellite data clearly shows that for poinsettia, you must detect the "right" markers (Moyer Declaration, para. 14). Unexpectedly, the present inventors have determined that SSR markers are not useful indicators of poinsettia cultivar identity and relationship. Likewise, there was absolutely no way that one of ordinary skill in the art could have known prior to the present invention that AFLPs would detect the "right" markers and

could be used to study genetic relationships among poinsettia cultivars. There is simply too much unpredictability regarding the presence of polymorphisms within the narrow poinsettia gene pool. According to the outstanding rejection, one would have expected that microsatellites would be as good as, if not better, than RAPD and AFLP to study genetic relationships in poinsettias. In fact, this is not the case. The failure of microsatellite analysis and the narrow genetic base indicates that new fingerprinting methods will need to be evaluated on a case-by-case basis in poinsettia (Moyer Declaration, para. 15) because success with one method (e.g., RAPD) is not at all predictive of success with other methods (e.g., microsatellites or AFLP).

Thus, the teachings of Ling et al. in view of Barcaccia et al. as defined by Dice would have provided neither the motivation nor reasonable expectation of success to one of ordinary skill in the art with respect to the present rejection, both of which are legally required to maintain the outstanding rejection.

Finally, with respect to claims 3-11, these claims as examined recite a "method of estimating a genetic relationship of a first poinsettia plant to a poinsettia plant that is a representative member of a specific breeding family." In other words, claims 3-11 are directed to methods of evaluating the breeding history or pedigree of a plant. As previously addressed in Applicants' last response it was unexpected that AFLP genetic fingerprints would track breeding history/pedigree of a wide range of poinsettia cultivars and that the different breeding families would produce closely related fingerprints (see *also*, Moyer Declaration, para. 7). The cited references are completely silent regarding the use of fingerprint analysis to evaluate breeding history (e.g., to determine whether a plant is "essentially derived" from a known cultivar) and do not in any way suggest this embodiment of the invention. Claim 3 has been amended herein to read "a method of assessing the breeding history of a first poinsettia" to more clearly recite this aspect of the invention, without narrowing the scope of the claimed invention. Accordingly, in addition to the reasons discussed above, the subject matter of claims 3-11 is further nonobvious over the cited art on the basis that none of the cited references, taken alone or in any combination, disclose or suggest a method of assessing breeding history of a poinsettia plant.

In view of the foregoing, Applicants respectfully submit that the claimed subject matter is nonobvious over Ling et al. in view of Barcaccia et al. as defined by Dice, and request that the outstanding rejection under §103(a) be withdrawn.

**II. Claims 1, 3, 5-7, 21, 23-24, 30, 63 and 69 are Patentable over Ling et al. in view of Sukhwinder et al. as Defined by Dice.**

The Final Action has maintained the rejection of claims 1, 3, 5-7, 21, 23-24, 30, 63 and 69 as unpatentable under §103(a) over Ling et al. in view of Sukhwinder et al. as defined by Dice. The Ling et al. reference has been addressed in detail above and in the Moyer Declaration. The deficiencies of Ling et al. are not remedied by the teachings in Sukhwinder et al. concerning rice or the analytical methods of Dice. The AFLP work in rice reported by Sukhwinder et al. is not relevant to poinsettias, and would not have provided the motivation or reasonable expectation of success with respect to the claimed invention that are legally sufficient to maintain the present rejection. In view of the unpredictability of genetic fingerprinting in poinsettia, the use of AFLPs in poinsettias would not have been at all obvious to one of ordinary skill in the art prior to the present invention (Moyer Declaration, para. 15). Further, a method of assessing the breeding history of a poinsettia plant using AFLP analysis is not in any way disclosed or suggested by Ling et al., Sukhwinder et al. or Dice, taken alone or in any combination.

Finally, the Final Action requests that the Park and Moyer manuscript be resubmitted accompanied by a declaration or affidavit. This manuscript has now published (Park and Moyer, *J. Amer. Soc. Hort. Sci.* 129:863-869, 2004), and a copy of this publication is enclosed. Applicants do not believe it is necessary to submit a publication with an accompanying declaration as the statements therein have been subjected to peer review ("Publications may, however, be evidence of the facts in issue and should be considered to the extent that they are probative"; MPEP § 716.02(g)), but would be happy to do so if such a submission would expedite the allowance of the present application.

In light of the discussion above, it is therefore respectfully requested that the obviousness rejection over Ling et al. in view of Sukhwinder et al. as defined by Dice be withdrawn.

**III. Claims 1, 3, 5-7, 21, 23-24, 30, 63 and 69 are Patentable over Ling et al. in view of Barker et al. as Defined by Tulloss.**

The Final Action has also maintained the rejection of claims 1, 3, 5-7, 21, 23-24, 30, 63 and 69 as unpatentable under §103(a) over Ling et al. in view of Barker et al. as defined by Tulloss. The Ling et al. reference has been addressed in the preceding two sections and in the Moyer Declaration. The teachings of Barker et al. regarding willow and/or the analytical methods of Tulloss et al. do not remedy the deficiencies of the Ling et al. reference. Again, the AFLP work in willow reported by Barker et al. is not relevant to poinsettias, and would not provide the requisite motivation or reasonable expectation of success with respect to the present invention. In view of the unpredictability of genetic fingerprinting in poinsettia, the use of AFLPs in poinsettias could not have been at all obvious to one of ordinary skill in the art prior to the present invention (Moyer Declaration, para. 15). Further, a method of assessing the breeding history of a poinsettia plant using AFLP analysis is not in any way disclosed or suggested by Ling et al., Barker et al. or Tulloss, taken alone or in any combination.

Accordingly, it is submitted that the present invention is patentable over Ling et al. in view of Barker et al. as defined by Tulloss, and request that the outstanding rejection under §103(a) on this basis be withdrawn.

**IV. Conclusion.**

The concerns of the Examiner having been addressed in full, Applicant respectfully requests withdrawal of all outstanding rejections and the issuance of a Notice of Allowance forthwith. The Examiner is encouraged to address any questions regarding the foregoing to the undersigned attorney, who may be reached at (919) 854-1400.

In re: Moyer et al.  
Serial No.: 09/912,072  
Filed: July 24, 2001  
Page 14 of 14

Respectfully submitted,



Karen A. Magri  
Registration No. 41,965

Enclosures:

Parks and Moyer  
Moyer Declaration

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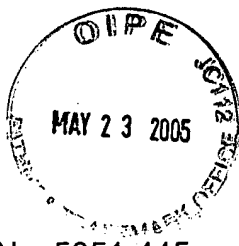
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Attorney's Docket No. 5051-445

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Moyer et al.  
Serial No.: 09/912,072  
Filed: July 24, 2001  
For: IDENTIFICATION OF POINSETTIA CULTIVARS

Examiner: M. Sheinberg  
Group Art Unit: 1634

**Declaration of Dr. James W. Moyer under 37 C.F.R. § 1.132**

I, James W. Moyer, do hereby declare and state as follows:

1. I am a Professor of Plant Pathology and Head of the Department of Plant Pathology at North Carolina State University. I received my undergraduate degree in Agronomy at Washington State University. I did my masters and doctoral work in plant pathology at The Pennsylvania State University. One of the research areas on my laboratory is a floral crop program that has focused on techniques for development of reliable fingerprinting technology as an aid to cultivar identification and more recently the development of tools for molecular marker assisted breeding.

2. I am a named inventor on U.S. Patent Application No. 09/912,072 (*hereinafter* "the '072 application").

3. I have read the following publications cited by the Examiner in connection with the '072 application:

- Ling et al., *HortScience* 32:122-124 (1997)
- Barcaccia et al., *J. Horticultural Science & Biotechnology* 74:243-250 (1999)
- Singh et al., *Crop Improv.* 25:15-20 (1998) (*referred to as "Sukhwinder et al."*)
- Barker et al., *Genome* 42:173-182 (1999)

4. Ling et al. concerns the use of RAPD techniques to compare the DNA of nine commercial poinsettia cultivars, which were from widely differing groups. Thus, the RAPD analysis of Ling et al. did not have to be robust to distinguish these cultivars. Singh et al., Barker et al., and Barcaccia et al. applied AFLPs to compare the fingerprints of cultivars from rice, willow and *Pelargonium* (geranium), respectively.

5. Prior to the work described in the '072 application, it would not have been obvious from Ling et al. that AFLP analysis of poinsettias could distinguish and estimate genetic relationships among different cultivars. AFLP analysis had been used in other plants, primarily crop plants such as rice and willow as described in the Singh et al. and Barker et al. publications. Some recent work with AFLPs had been reported in ornamental plants including *Pelargonium* as described by Barcaccia et al.



However, it simply was not known whether AFLP analysis would be able to detect sufficient inter-cultivar polymorphisms among poinsettia cultivars. Barcaccia et al.'s work with geranium would not have been predictive with respect to poinsettia, because the gene pools of these plants are distinct.

6. Poinsettia is an asexually reproducing species, with a narrow genetic base. Most poinsettia cultivars have been identified by selection of sports or induced mutations. As a result, there is very little pedigree information available for poinsettia. Prior to the invention described in the '072 application, it was uncertain whether there would be sufficient genetic polymorphisms detectable by AFLP among poinsettia cultivars.

7. Further, it was not at all obvious in advance that the polymorphisms and genetic fingerprints would be powerful enough to track the breeding history or pedigree of a broad range of poinsettia cultivars and that the different breeding families would have distinct and closely related fingerprints. One of the exciting discoveries that came out of the work described in the '072 application was the finding that differences in the AFLP fingerprints were reflective of breeding lineage. While there is speculation in the literature about relationships, none of the cited publications (Ling et al., Singh et al., Barker et al., Barcaccia et al.) demonstrate this relationship between polymorphism and breeding history.

8. The unpredictability in fingerprinting methods as applied to poinsettia is also evident in our work with microsatellites. We have tried to evaluate genetic relationships among poinsettia cultivars using microsatellite simple sequence repeat (SSR) analysis. Microsatellites are sections of DNA composed of repeats of short motifs (e.g., CA, GTG, TGCT, etc.) arranged in tandem. The sequence surrounding the repeat region is usually conserved, allowing amplification primers to be designed so that the repeat region and a short flanking sequence can be amplified. Polymorphisms are observed in the number of repeats present.

9. Approximately 700,000 bases of poinsettia sequence were obtained from a genomic library constructed of partially digested 'Freedom Pink' DNA. Using a computer algorithm, the sequence was scanned for the presence of SSR motifs of significant size to be polymorphic based on previous studies (Cardle et al., *Genetics* 847-854 (2000); Alvarez et al., *Theor. Appl. Genet.* 103:1283-1292 (2001)). The minimum number of repeats selected were 6 for dinucleotide motifs, 5 for trinucleotide motifs, 4 for tetranucleotide motifs, and 4 for pentanucleotide motifs. As far as I am aware, no other studies have reported sextanucleotide motifs; the minimum number of repeats for this motif was set at 3. A total of 20 SSR motifs were isolated. They consisted of: 11 dinucleotide, 6 trinucleotide, 1 tetranucleotide, 1 pentanucleotide, and 1 sextanucleotide. Primers were designed for 18 of the 20 SSRs; two of the motifs were near the end of the cloned insert such that there was not sufficient flanking region in which to design a primer.

10. The plant material selected for evaluation consisted of 48 cultivars of poinsettia representing 12 of the major cultivar groups of color sports, as well as 4

other cultivars. These groups included Angelika, Annette Hegg, Celebrate, Cortez, Freedom, Gross, Lilo, Nutcracker, Pepride, Peterstar, Sonora, and V14 Glory. The 4 additional cultivars selected were 'Winter Rose', 'Pearl', 'Prestige', and 'Snowcap'. Duplicate samples of 2 cultivars taken from different plants were used as controls.

11. Twelve of the primer pairs amplified a fragment of the predicted size, whereas the rest could not be optimized and either did not amplify or resulted in too complex a pattern to evaluate. The primer pairs amplified from 1-5 alleles each, with an average of 2 alleles. Three primer pairs amplified one allele. Six of the loci were polymorphic, with 2 to 4 alleles. Duplicate cultivars consistently amplified the same number of alleles.

12. Statistical analysis of the data revealed a narrow range of distances and low resolution of cultivars and cultivar groups on a dendrogram (Appendix 1; attached). Shared allele distances ranged from 0-0.25. The largest distance, 0.25 was between the: Hegg group and V14 Glory Red; V14 Glory Pink/V14 Glory White and Pepride Red, Winter Rose, and the Cortez group; Winter Rose and the Lilo group; and the Sonora group and the Lilo group. Many cultivar comparisons had a distance of 0 and could not be differentiated, as seen in the attached dendrogram. Some cultivar groups could be differentiated from each other and formed unique clusters on the dendrogram; Hegg, Cortez, Sonora, and Lilo cultivar groups formed unique clusters with a distance of 0. Other cultivar groups were divided or clustered with unrelated groups. The pink and white cultivars of the Celebrate 2 and V14 Glory groups each formed unique clusters separate from the Red "parent" cultivars of these groups. The white cultivars Angelika White, Snowcap, Nutcracker White, and Pearl clustered together with a distance of 0. Finally, a large cluster with a 0 distance was made up of the Freedom, Peterstar, and Gross groups as well as the Angelika and Nutcracker groups minus the white cultivars.

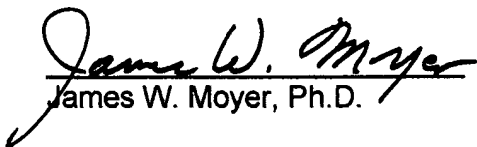
13. As the results described above demonstrate, the power of microsatellite techniques to differentiate cultivars was not evident in poinsettia, likely due to the narrow genetic base of this crop resulting from the methodologies used in poinsettia breeding programs, which rely heavily on mutation breeding and selection of sports. This finding is unexpected; based on the known properties of microsatellites, I would have expected this approach to have worked as well, or even better, than AFLPs in distinguishing poinsettia cultivars (see, e.g., Margante et al., *Plant J.* 3:175-182 (1993); Maguire et al., *TAG* 104:388-398 (2002). It is known that SSR markers tend to have a higher level of heterozygosity than AFLP markers due to codominance of SSR loci. In addition, SSR markers generally have greater somatic stability than AFLP markers. Finally, SSR techniques are typically found to be more technically reproducible than AFLP. However, from our data, it appears that the narrow genetic base of poinsettia lacks polymorphisms in the SSR loci.

14. RAPD, AFLP and SSR markers are each distinct. RAPD markers detect polymorphisms based on hybridization of short primers to random locations in the genome, whereas AFLP markers detect polymorphic restriction sites in the genome or at least polymorphisms in close proximity to restriction sites.

Microsatellites detect polymorphisms in the number of short tandem motif repeats that are present in the genome. RAPD, AFLP and SSR would therefore each detect a different subset of polymorphisms. The microsatellite data discussed above demonstrates that for poinsettia you must detect the "right" polymorphisms in order to distinguish among poinsettia cultivars.

15. The narrow genetic base of poinsettia and the failure of microsatellite analysis to distinguish poinsettia cultivars indicate that there is unpredictability in the application of fingerprinting techniques to poinsettia, with each approach needing to be evaluated on a case-by-case basis. It therefore would not have been obvious prior to the experimentation described in the '072 application that sufficient AFLP polymorphisms would be present in the poinsettia gene pool for AFLPs to be successful in distinguishing and determining genetic relationships among poinsettia cultivars.

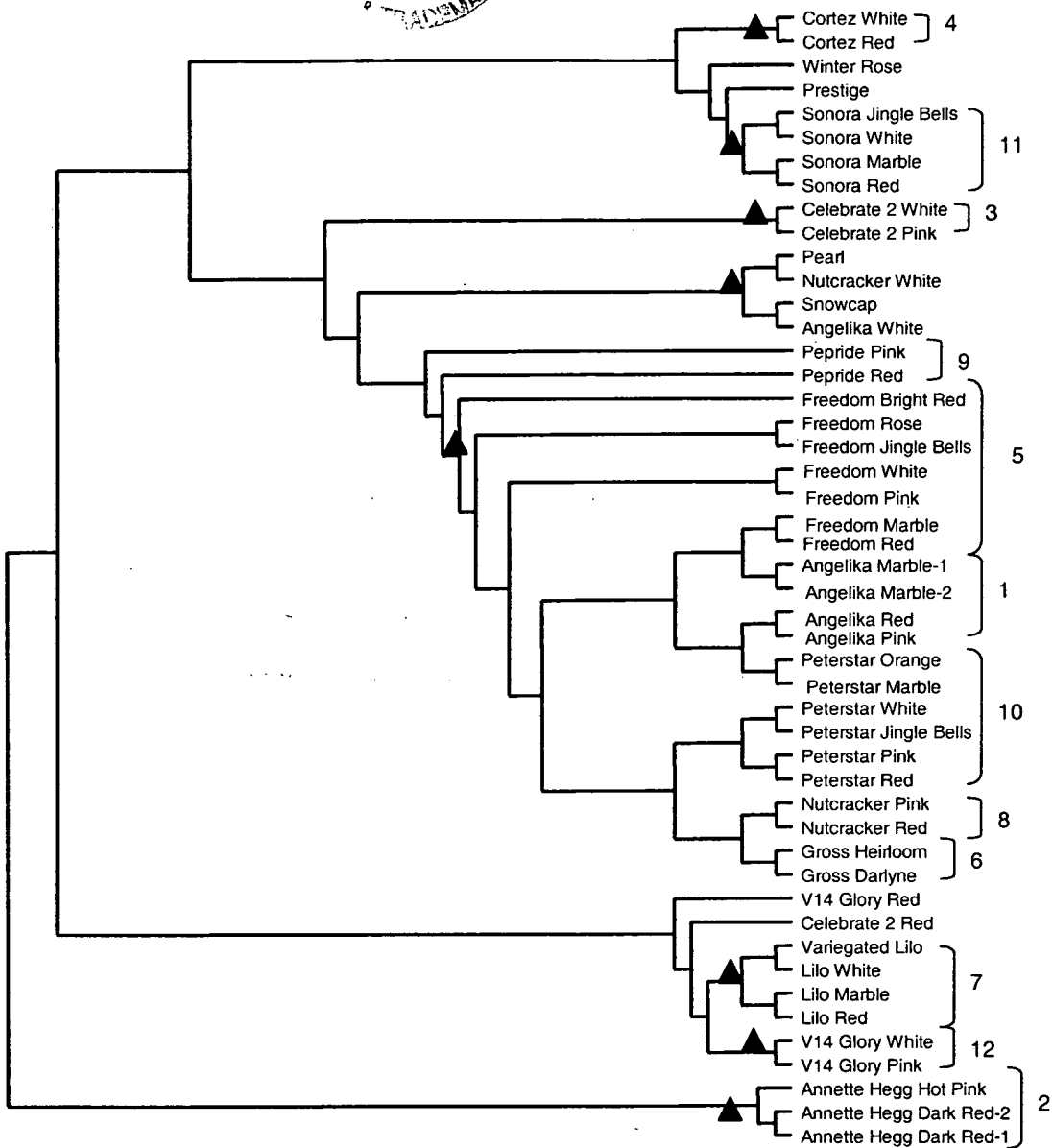
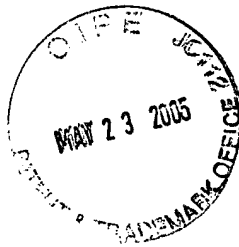
16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

  
James W. Moyer, Ph.D.

5/19/05  
Date

Attachment: Appendix A

# APPENDIX I



Dendrogram of 44 poinsettia cultivars and 2 duplicates generated with microsatellite data using shared allele distance and Neighbor-Joining clustering. Brackets and numbers denote cultivar groups. Triangles denote clusters of cultivars with a distance of 0.

# Evaluation of AFLP in Poinsettia: Polymorphism Selection, Analysis, and Cultivar Identification

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**ADDITIONAL INDEX WORDS.** DNA, floral crop, intracultivar variation, fingerprinting, genetic analysis, molecular differentiation

**ABSTRACT.** Fingerprinting using molecular markers is a highly effective method of cultivar identification that is a powerful aid to traditional methods based on morphology. Amplified fragment length polymorphism (AFLP) is a robust and reliable method for generating molecular markers that has been used to evaluate many crops for a variety of applications. In this study, AFLP was used to develop and validate robust genetic fingerprints for poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzch) cultivars. Polymorphism selection was completed to facilitate the identification of useful polymorphisms and minimize future fingerprinting costs and time. Poinsettia is a highly variable crop subject to genetic drift and variable cultivars. Validation of polymorphisms to remove those associated with intracultivar variation improved the reliability of the fingerprinting. The result was a poinsettia AFLP database that defines the genetic fingerprints of 104 cultivars. Cluster analysis illustrated differentiation of most poinsettia cultivars tested. Selection of a subset of AFLP polymorphisms resulted in clustering of cultivars according to known origin and breeding program. This method has applications not only for cultivar identification for cultivar protection, and maintenance of cultivar uniformity, but also has the potential application of developing markers for important traits.

Poinsettia is the most valuable potted plant in the United States, with a wholesale value of \$256 million in 2001 (USDA, 2003). Poinsettias were introduced to the United States in the early 1800s, yet the first commercial-quality cultivars were not introduced until 1963. Today, more than 175 cultivars of cultivated poinsettia are available. Because of the ever-changing selection of cultivars and their valuable market share, breeders are under intense pressure to develop new cultivars. With this process comes the desire to protect the cultivars and breeder's rights. Evaluation of morphological characteristics, such as bract color, growth habit, and time to flower, has been the primary method of cultivar identification; however, there are several shortcomings to this method. Morphological characteristics may be similar between some cultivars, making differentiation difficult. These traits are also influenced by environmental conditions, which can cause variation in their appearance. Finally, morphological evaluation can be costly, as plants must be maintained for an entire growth cycle to score many of these traits.

Molecular techniques have distinct advantages over morphological evaluation for cultivar identification. They are not influenced by environmental factors, making them more reliable and stable. These methods can be applied at almost any stage of growth, reducing the time and cost of cultivar identification. Molecular techniques can provide more genetic information, since the number of molecular markers is virtually unlimited. A limited number of molecular studies have been done with floral crops. Fingerprinting of petunia (*Petunia hybrida* L.) has been reported using restriction fragment length polymorphism (RFLP) (Beyermann et al., 1992; Vainstein and Ben-Meir, 1994) and randomly amplified polymorphic DNA (RAPD) (Peltier et al., 1994). Microsatellites have been used for molecular analysis of rose (*Rosa hybrida* L.) (Esselink et al., 2003). Recently, methods such as RAPD (Ling et al., 1997), DNA amplification

fingerprinting (DAF) (Starman and Abbitt, 1997), and arbitrary signatures from amplification profiles (ASAP) (Starman et al., 1999) have been applied to poinsettia to provide faster and more definitive methods of cultivar differentiation. However, these studies included a limited number of cultivars (9, 11, and 11, respectively) and did not assess the variability of (validate) each polymorphism, nor was the method tested across diverse as well as closely related cultivars.

Amplified fragment length polymorphism has distinct advantages over other molecular techniques. AFLP detects polymorphisms throughout the genome by selective amplification of restriction fragments, rapidly producing a large number of markers. AFLP is highly reproducible (Jones et al., 1997) and requires no prior genetic information (Vos et al., 1995). Exploitation of the AFLP technology could provide increased resolution capable of differentiating the more than 175 existing poinsettia cultivars. In addition, this database could be used to generate estimates of similarity between candidates and existing cultivars.

Research with other crops has shown the utility of AFLP for determining relationships between cultivars and for cultivar identification, including bermudagrass (*Cynodon* L.C. Rich.) (L.H. Zhang et al., 1999), eggplant (*Solanum* L.) (Mace et al., 1999), and lettuce (*Lactuca* L.) (Hill et al., 1996). Recently, AFLP has been applied to ornamental crops for the same purpose, including geranium (*Pelargonium* L'Hér.) (Barcaccia et al., 1999), peruvian lily (*Alstroemeria* L.) (Han et al., 1999, 2000), rose (*Rosa* L.) (D. Zhang et al., 1999), daylily (*Heimerocallis* L.) (Tomkins et al., 2001), and new guinea impatiens (*Impatiens hawkeri* W. Bull.) (Carr et al., 2003; J.H. Lysterly, unpublished data). Generally, these studies show that genetic marker data agree with available pedigree data (Carr et al., 2003).

The objectives of this study were to optimize the use of AFLP as a fingerprinting tool for poinsettia and to determine the extent of variability detected with specific AFLP polymorphisms. In this study we have used AFLP to identify a set of polymorphic DNA fragments that are useful for poinsettia cultivar differentiation. The polymorphisms were selected based on their consistent presence or absence in replicates of selected cultivars. In addition,

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<sup>1</sup>To whom reprint requests should be addressed.

multiple statistical models were evaluated for use in generating similarity or dissimilarity indices that would facilitate genotype comparisons.

## Materials and Methods

**PLANT MATERIAL.** One hundred and four poinsettia genotypes and two other *Euphorbia* L. species, *E. fulgens* Karw. ex Klotzch and *E. cornastra* (Dressler.) Radcl.-Sm., were included in the fingerprint analysis. For the validation study, 77 sources of nine genotypes, as shown in Table 1, were collected from locations worldwide and coded to remove analytical bias. Plant material was provided by breeders or collected from two poinsettia trials conducted at the Horticulture Field Laboratory, North Carolina State University.

Genomic DNA was isolated from fresh tissue using a modified benzyl chloride procedure (Zhu et al., 1993). The extraction protocol was adapted to small volume processing of 150 mg of leaf tissue in a microcentrifuge tube. Fully expanded leaves with midribs removed were selected from all cultivars for extraction.

**AFLP ANALYSIS.** The AFLP protocol was performed as described by Vos et al. (1995) using AFLP Analysis System I (Life Technologies, Gaithersburg, Md.) with the following modifications. The Life Technologies protocol was modified by extending the restriction digest incubation to overnight and increasing the ligation incubation to 6 h at 16 °C to improve reproducibility in the final AFLP result.

Table 1. Poinsettia cultivars used to identify polymorphisms useful for fingerprinting and cultivar identification. Group 1 cultivars were used to identify AFLP primers useful for cultivar identification. Group 2 cultivars were used to evaluate intracultivar variation of the polymorphisms and to validate the poinsettia AFLP fingerprints.

Cultivar	No. of sources
<i>Group 1 cultivars</i>	
Bonita	1
Freedom Jingle Bells	1
Freedom White	1
Gross Heirloom	1
Peterstar Jingle Bells	1
Peterstar Pink	1
Maren	1
Angelika Red	1
Freedom Red	3
Peterstar Red	1
<i>Group 2 cultivars</i>	
Angelika Red	7
Freedom Red	13
Peterstar Red	8
Hegg Dark Red	5
Supjibi	6
Lilo Red	5
Snowcap	8
Sonora Red	19
V-14 Glory Red	6

<sup>32</sup>P was used to label the *Eco*RI primer for specific amplification. The products of the specific amplification were electrophoresed on a 6% denaturing acrylamide gel at 60 W for ≈2 h. The gel was fixed in 5% acetic acid/5% methanol and dried in a gel dryer (BioRad, Hercules, Calif.), then exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, Calif.) overnight. The screen was then scanned on a phosphorimager (Molecular Dynamics) and the gel image was saved as a TIFF file.

**DATA ANALYSIS.** The gel image was analyzed using Pro-RFLP image analysis software (DNA ProScan, Nashville, Tenn.);  $\phi$ -X174/*Hinf*I (Promega, Madison, Wis.) was used as the standard reference to size the AFLP fragments. Selected AFLP polymorphisms were sized and scored as present (1) or absent (0). The scored data was exported as 1 or 0 to a Microsoft Excel (Microsoft, Inc., Redmond, Wash.) spreadsheet. Coefficients of association, for both similarity and distance, were generated from the binary data using five different models, three that incorporate only positive matches (1/1), and

two that incorporate both positive and negative matches (0/0). Distance was calculated using a model described by Lynch (1988). Similarity and all other analyses were calculated using four different coefficients in the statistical software package NTSYSpc 2.0 (Exeter Software, Setauket, N.Y.): 1) Dice,  $S_{ij} = 2a/(2a + b + c)$ , where  $S_{ij}$  is the similarity between two cultivars,  $i$  and  $j$ ,  $a$  is the number of fragments shared by  $i$  and  $j$ ,  $b$  is the number of bands present in  $i$  and absent in  $j$ , and  $c$  is the number of bands present in  $j$  and absent in  $i$  (Dice, 1945); 2) Jaccard,  $S_{ij} = a/(a + b + c)$  (Jaccard, 1908); 3) simple matching,  $S_{ij} = a + d/a + b + c + d$ , where  $d$  is the number of bands absent in both  $i$  and  $j$  (Sokal and Michener 1958); and 4) unnamed coefficient 1,  $S_{ij} = 2(a + d)/2a + b + c + 2d$ . The similarity or distance matrices were then analyzed using four different SAHN clustering methods, UPGMA (unweighted pair-group method; Sokal and Michener, 1958), WPGMA (weighted pair-group method; Sneath and Sokal, 1973), CL (complete linkage; Lance and Williams, 1967), and SL (single linkage; Lance and Williams, 1967). Dendrograms were created from the clustered matrix using TREE. The COPH and MXCOMP programs calculated the goodness of fit of the clustering to the data matrix. Principal coordinates analysis was performed using DCENTER and EIGEN.

## Results

**SELECTION OF POLYMORPHISMS.** A two-level screening strategy was used to determine which AFLP primer pairs were the most appropriate for fingerprinting poinsettia cultivars. Initially, all 64 possible primer combinations in AFLP Analysis System I were used to amplify DNA from four poinsettia genotypes (C1, C17, C27, and Selection 119) that were the non-grafted progenitors of three major cultivar groups. The phytoplasma-free genotypes were selected to insure that the polymorphisms were poinsettia in origin, and to span the diversity of poinsettia cultivars. Primer combinations were ranked based on the number, intensity, and reproducibility of polymorphisms. Polymorphisms were selected for analysis if they were present in at least one phytoplasma-free cultivar, easily scored on the AFLP image in terms of intensity and separation from other fragments, and reproducible in at least two independent amplifications. A preliminary evaluation of the four best primer combinations on a larger number of cultivars, listed as 1-4 in Table 2, did not result in a high level of differentiation.

Table 2. AFLP primer combinations selected for fingerprint analysis of poinsettia cultivars. Average includes the total number of fragments ranging from 60 to 750 bp. Scored polymorphisms are all polymorphisms scored prior to validation. Cultivar-linked polymorphisms are validated polymorphisms used in the final analysis. E = GACTGCGTACCAATTC and M = GATGAGTCCTGAGTAA.

Primer combination	Avg no. of fragments	Scored polymorphisms (no.)	Cultivar-linked polymorphisms (no.)
1 E-AAG/M-CTA	99	10	5
2 E-AAG/M-CTG	91	18	9
3 E-ACA/M-CTC	95	8	4
4 E-ACA/M-CTT	109	10	4
5 E-ACA/M-CTA	88	11	4
6 E-ACA/M-CTG	70	6	2
7 E-AGC/M-CAC	55	21	7
8 E-AGC/M-CTA	71	14	6
Total		98	41

A second evaluation of the 30 best primer combinations from the initial screen was completed with a set of 12 cultivars spanning a broad range of similarities, including three sources of the same cultivar from different sources for detecting intracultivar variation, listed as Group 1 in Table 1. The primer combinations that generated the most useful polymorphisms were scored and analyzed individually, and prioritized according to their ability to detect polymorphisms between closely related cultivars without detecting intracultivar variation. The new primers were then analyzed in combinations with the original four primers according to priority, until the similarities for the set of cultivars were optimized; this balanced differentiation of cultivars with clustering of related cultivars. Four additional primer combinations were selected, listed as 5-8 in Table 2. A total of 98 AFLP polymorphisms were selected for further analysis, resulting in Jaccard similarities ranging from 0.27 to 0.98.

**REPRODUCIBILITY OF POLYMORPHISMS.** To further validate the reproducibility and reliability of the AFLP fingerprints, the polymorphisms were evaluated. In a preliminary study to test the hypothesis that some polymorphisms were in regions of the genome associated with intracultivar variation, we analyzed plants from multiple sources of five cultivars: 'Freedom Red' (seven sources), 'Hegg Dark Red' (two sources), 'Peterstar Red' (two sources), 'Lilo Red' (two sources), and 'V14 Glory Red' (one source). The results of this test demonstrated the presence of intracultivar variation among the polymorphisms.

In a larger test designed to identify the polymorphisms that should be excluded from the analysis, we used 77 sources of nine different cultivars as listed in Table 1. The amount of intracultivar variation of the polymorphisms was different for each of the cultivars. 'Angelika Red' showed the least variation with differences noted in 10 of the 98 polymorphisms. Sources from four of the validation cultivars, 'Freedom Red', 'V14 Glory Red', 'Snowcap', and 'Hegg Dark Red', showed the most variation. One source of each of these cultivars was very different from all other sources of the cultivar; differences were seen in as many as 33 of the 98 polymorphisms. Polymorphisms that were variable among sources of a given cultivar were likely to be variable among sources of one or more other cultivars; 22 varied in one cultivar, and 35 varied in more than one. Of the 98 polymorphisms, eight were consistent in all sources of all cultivars. In total, 57 of the 98 polymorphisms were found to be highly variable, varying in more than one source of a particular cultivar or cultivars. These highly variable polymorphisms were eliminated from AFLP analysis. The 41 validated polymorphisms were used to create a poinsettia AFLP database for poinsettia genotypes and two outgroup species, *E. cornastra* and *E. fulgens*. This database includes 81 commercially released cultivars as well as 23 unnamed, unreleased genotypes of poinsettia.

**DATA ANALYSIS.** To determine which statistical methods would yield the most accurate representation of the relationships between the cultivars in this study, comparisons were made between five similarity or distance coefficients and four clustering methods. The dendrograms constructed using the various association and clustering methods were examined and the cophenetic correlation coefficients of each were compared (Table 3), to test the goodness of fit of the association coefficient to its respective dendrogram. The association coefficients that gave the highest cophenetic correlation coefficients were those that incorporated only positive matches (1/1). UPGMA clustering gave the highest correlation coefficients of the clustering methods, from 0.813 to 0.877, indicating a good fit of the similarity matrix to the dendrogram.

The coefficients that incorporated only positive matches, Dice, Lynch, and Jaccard, yielded the same cultivar group clusters in the same orientation as shown in the dendrogram of 81 named cultivars and two outgroup species (Fig. 1). The two coefficients that incorporated positive and negative matches, SM and UN1, also clustered the cultivar groups, but the orientation of the clusters on the dendrogram differed from that of the other three methods. Jaccard's similarity coefficient, when clustered with the UPGMA technique, gave the highest of all the correlation values, 0.877, indicating the best fit to the data.

There was a full range of Jaccard similarity coefficients. Between poinsettia cultivars, similarity coefficients ranged from 1 (identical), between 21 different cultivar pairs, and 0.219 (least similar), between 'Airbrush' and 'Freedom Rose'. The similarities between related cultivars ranged from 1 to 0.615, with the lowest similarity being between 'Freedom Red' and its color sport 'Freedom Rose'. *E. cornastra* had much lower similarities to the poinsettia cultivars, with coefficients ranging from 0.032 to 0.217. *E. fulgens* also had lower similarities to the poinsettia cultivars, ranging from 0.152 to 0.433. The similarity between these two outgroup species was 0.095.

A small set of the cultivar pairs could not be differentiated with this set of AFLP polymorphisms. The 21 identical pairwise comparisons involved 16 cultivars from three main cultivar groups; one comparison involved 'Lilo', seven were from 'Freedom', and 13 were from 'Angelika'/'Peterstar'. All of the cultivar pairs with identical coefficients were related; some of these cultivars are color sports resulting from either natural or induced mutations. Seven comparisons involved color variants of a cultivar and seven others were color variants of related cultivars. Four comparisons involved cultivars with a leaf variegation mutation. One comparison was between a cultivar and a selection of the same cultivar. The remaining two comparisons were also pairs of derivative cultivars, and had similar coloring of bracts and leaves.

Figure 1 shows the dendrogram of the 81 commercially released cultivars and the two *Euphorbia* outgroup species. A critical value of  $\approx 0.63$  resulted in five major clusters on the dendrogram: 'Lilo'/'Hegg', 'Cortez'/'Sonora', 'Peterstar'/'Angelika', 'Celebrate'/'V14 Glory', and 'Freedom'. The most distant poinsettia cultivar was 'Xenia Red Deluxe', which branched from the dendrogram at  $\approx 0.42$ . *E. fulgens* and *E. cornastra* made up the outermost branches at  $\approx 0.31$  and 0.12, respectively.

Principal coordinates analysis gave further support to the clusters created by UPGMA clustering. The first three eigenvectors explain 56.93%, 9.88%, and 4.09% of the total variation, cumulatively accounting for 70.91% of the variation. The three-dimensional PCO plot shows the same clusters found with SAHN analysis, 'Lilo'/'Hegg', 'Cortez'/'Sonora', 'Peterstar'/'Angelika', 'Celebrate'/'V14 Glory', and 'Freedom'.

**CULTIVAR GROUP ANALYSIS.** The genetic lineages of all cultivars were not available to provide direct evidence of a correlation

Table 3. Cophenetic correlation coefficients for the five similarity or distance coefficients and four clustering methods applied to the final AFLP data set containing the 81 commercial poinsettia cultivars and two outgroup species.

	Dice	Jaccard	SM	UN1	Lynch
UPGMA	0.870	0.877	0.839	0.813	0.867
WPGMA	0.855	0.861	0.807	0.775	0.817
CL	0.759	0.791	0.775	0.733	0.751
SL	0.785	0.778	0.754	0.726	0.781

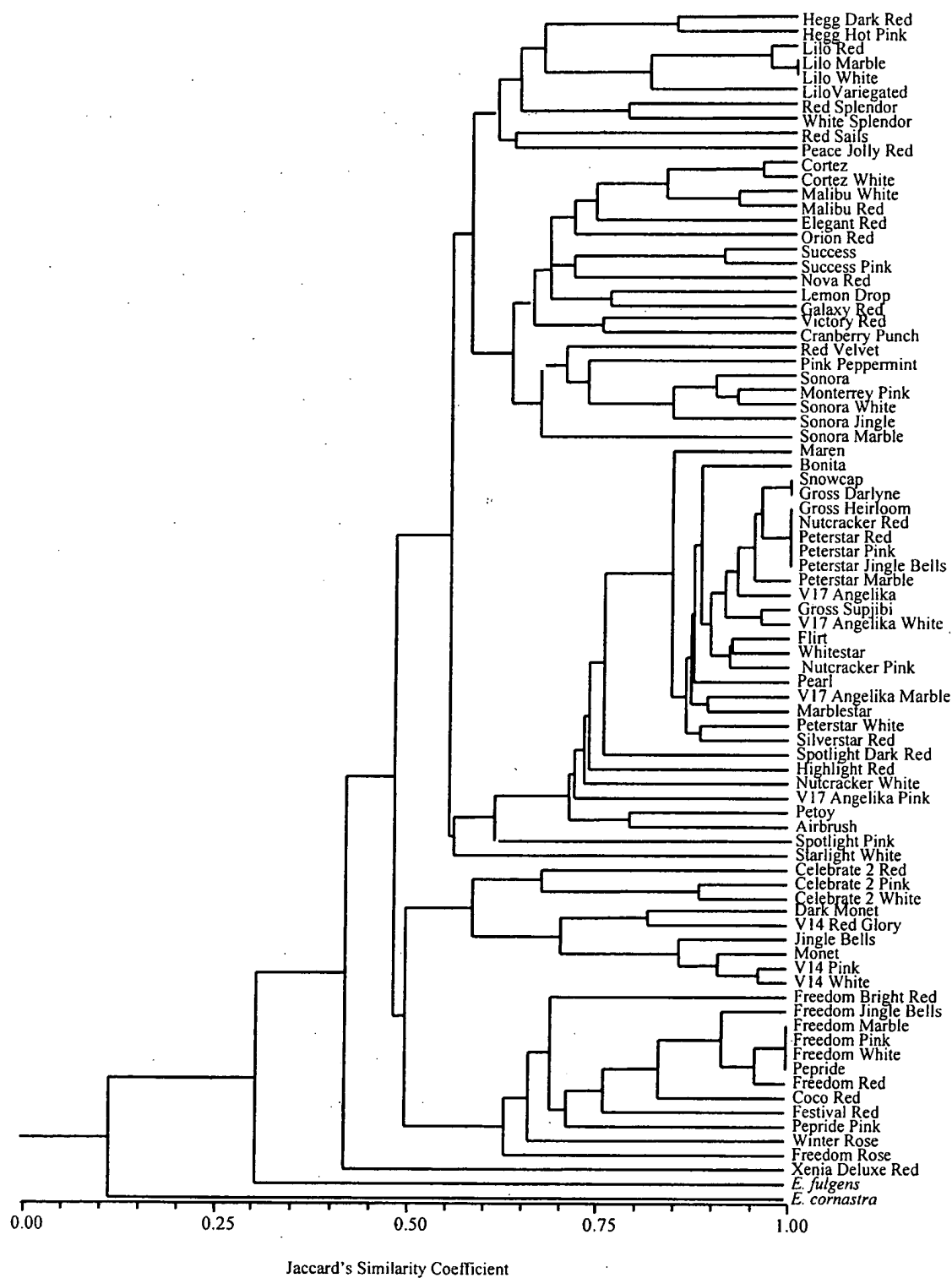


Fig. 1. Dendrogram of 81 commercial poinsettia cultivars and two outgroup species using 41 AFLP polymorphisms, generated using Jaccard's similarity coefficient and UPGMA clustering.



between polymorphic profile similarity and genealogy; furthermore, many cultivars are derived from natural or induced mutations arising from existing genotypes. Alternatively, an iterative strategy was derived to determine the extent that the measure of similarity of polymorphic profiles reflected genetic relationships and thus the probability that a cultivar originated from a specific breeding program. The strategy consisted of obtaining genetic information from available plant patents and grouping cultivars based on known origin and breeding program. A subset of 14 of the 41 polymorphisms present in all seven of the 'Freedom'-derived cultivars was initially selected as the basis of a strategy to identify the breeding program or origin of the genotypes. When this set of polymorphisms was used to generate a dendrogram, the 'Freedom'-derived cultivars clustered together with a similarity of 1, with other cultivar groups clustering in a similar manner to that on the dendrogram that included all of the AFLP polymorphisms. Examination of nine cultivar groups revealed within-group consistency of five of the 14 polymorphisms; within the 'V14 Glory', 'Freedom', 'Celebrate', 'Cortez', 'Sonora', 'Peterstar', 'Angelika', 'Lilo', and 'Hegg' cultivar groups, the polymorphisms were either consistently present or absent. In addition, two more that were absent in all 'Freedom' cultivars were consistently present or absent in the other nine cultivar groups. Using these seven polymorphisms, a new dendrogram was generated, which placed all of the genotypes sharing a common origin into clusters with similarities of 1 (Fig. 2). The cophenetic correlation of this dendrogram was 0.869, showing high correlation of the clustering to the data. Principal coordinates analysis supported the same clusters, with the first three eigenvectors explaining a total of 68.67% of the variation.

To test the significance of the subset of AFLP polymorphisms, a random set of seven polymorphisms was generated from the 41, similarities were calculated, and a dendrogram was generated. The majority of cultivar groups were only partially clustered, and clustering of completely unrelated cultivars occurred with this set of polymorphisms. This shows the selection of the set of AFLP polymorphisms for cultivar group analysis was not due to random chance; clustering was dependent on the particular polymorphisms and was not an artifact of the small number of polymorphisms used in the analysis.

## Discussion

The dendrogram of 81 commercially released cultivars constructed using the entire set of 41 validated polymorphisms is consistent with known pedigrees of poinsettia according to breeder and cultivar patent information. 'Hegg', 'Lilo', and 'Splendor' cultivars share a common ancestry, and they form a unique cluster on the dendrogram. 'Cortez' resulted from a cross of 'Lilo' and an unknown cultivar, and it occupies a cluster adjacent to 'Lilo'. 'Malibu Red' was developed from a cross involving 'Cortez', and clusters close to it. Mutation or natural breeding from 'Angelika Red' developed a number of cultivars that cluster in the center of the dendrogram. The three 'Celebrate' cultivars form the next cluster on the dendrogram. Cultivars that share a background of 'V-14', 'Jingle Bells', and 'Monet' form a distinct cluster. The 'Freedom' cluster includes the 'Freedom Red' color sports developed by mutation and natural breeding, as well as 'Coco Red', 'Festival Red', and 'Pepride', all developed from 'Freedom Red'. Finally, the outgroup species *E. fulgens* and *E. cornsata* occupy the outmost branches of the dendrogram.

The set of 41 AFLP polymorphisms was able to differentiate

all but 21 pairs of cultivars out of 3240 pairwise comparisons of the 81 cultivars. Although all cultivars appeared to be differentiated using the full set of 98 AFLP polymorphisms, removal of polymorphisms that were hypervariable reduced the resolution of the test, causing the 21 pairs of cultivars to no longer be differentiated. All but two of the cultivars that could not be differentiated with the set of polymorphisms are easily separated by morphological traits; 'Nutcracker Red' and 'Peterstar Red' were the only morphologically similar cultivars that could not be differentiated with this set of AFLP data. All other undifferentiated cultivars were either color sports of one another or had different leaf variegation. Most of those cultivars that are the most difficult to distinguish morphologically, such as the numerous red, white, and pink cultivars in the study, were differentiated with AFLP fingerprinting. This demonstrates the importance of validating polymorphisms, as some that appear to discriminate between sports of some poinsettia cultivars could potentially cause false identification of a cultivar if used in fingerprinting. Sports may be difficult to distinguish genetically (Weising et al, 1995), and a more sensitive method may be necessary to fully differentiate the cultivars.

Additional selection of polymorphisms facilitated reliable classification of the cultivars into clusters indicative of genetic background and the breeding program or origin of the cultivar. The core set of seven AFLP polymorphisms selected using color variants places the cultivars in tight clusters of cultivar groups. Obvious cultivar groups with shared names clustered as expected, into groups with a similarity of 1. Other groups on the dendrogram with a similarity of 1 appear to cluster several cultivar groups together, 'Angelika' and 'Peterstar' groups for example. Further investigation into the breeding history of these cultivar groups revealed common ancestry; clustering using the core set of polymorphisms was a good predictor of breeding history of the cultivars. This cultivar group AFLP analysis could be used as a preliminary identification tool to place cultivars in the correct cultivar group, or it could be used to identify breeding group for cultivar protection purposes.

Other AFLP studies (Carr et al., 2003; Han et al., 2000) have focused on scoring a large number of polymorphisms to explain expected relatedness. However, this study shows that it is the quality as well as quantity of information that the polymorphisms contain, and not the quantity of polymorphisms themselves, which explains relationships and creates AFLP fingerprints. Selection of appropriate polymorphisms through validation leads to a robust system for analysis of relationships and creation of an AFLP fingerprint. The process is similar to validation of polymorphisms for any set of complex traits; here it is cultivar identification. Optimization of the set of polymorphisms can provide any level of differentiation that is needed. To help determine the breeding origin of a cultivar, the group-specific data can be used, and to differentiate, or fingerprint, a particular cultivar, the entire set of polymorphisms should be used. In addition, minimizing the number of primer combinations reduces the cost and time required to fingerprint cultivars. A high percentage of the expected relatedness can be explained by selecting the optimal primer combinations. In one other example, Ellis *et al.* (1997) found that by selecting the six best primer combinations, more than 80% of the expected relatedness could be explained. This study selected the best eight primer combinations from 64 possible, so it is possible to conclude that this study has found the majority of expected relatedness.

Other AFLP studies to date, especially those in ornamentals,

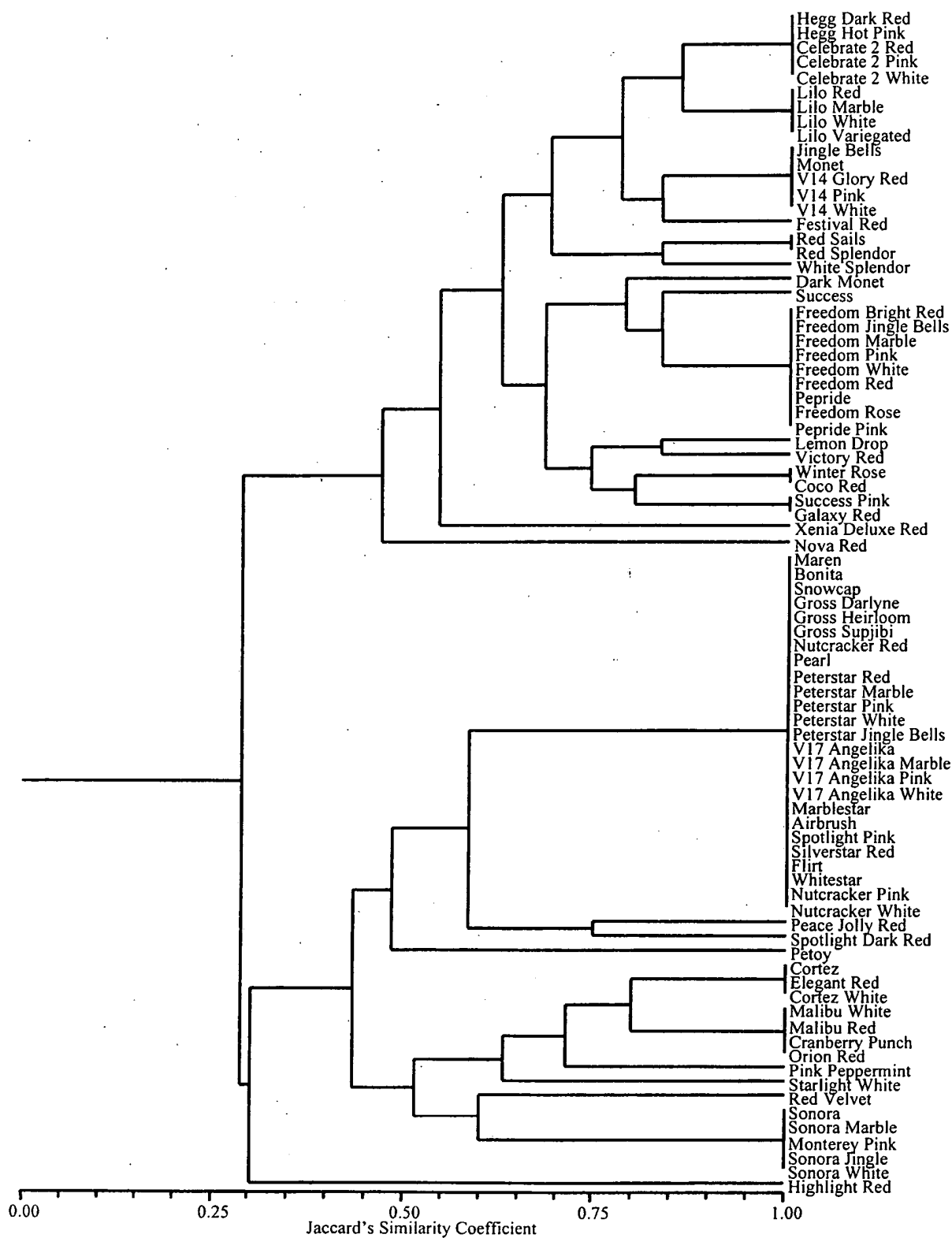


Fig. 2. Dendrogram of 81 commercial poinsettia cultivars using seven cultivar group specific AFLP polymorphisms, generated using Jaccard's similarity coefficient and UPGMA clustering. Those cultivars with a similarity of 1, represented in the dendrogram with a vertical line, denote cultivar groups.

have not addressed intracultivar variation of polymorphisms (Carr et al., 2003; Ling et al., 1997; Starman and Abbitt, 1997; Starman et al., 1999; D. Zhang et al., 1999). However, this study shows that analysis of the intracultivar variation of AFLP polymorphisms is vital to determine the degree of variation in the species of interest. Poinsettia is a vegetatively propagated crop that must be selected annually to maintain crop uniformity, demonstrating the variability that exists in this crop. Once the degree of intracultivar variation is established, some method to compensate for it should be implemented to establish a more robust fingerprint. The variation of specific AFLP polymorphisms in several different cultivars suggests that some of them likely originate from highly variable regions of the genome, and should be eliminated from fingerprint analysis. Discriminating between those polymorphisms that are reflective of this heterogeneity and those that are stable and connected with the distinct nature of that cultivar is essential to generating a reliable fingerprint. In addition, the high degree of intracultivar AFLP variation in poinsettia suggests that a molecular tool such as AFLP would be valuable in maintaining homogeneity of cultivar when used for marker-assisted breeding.

An additional important factor that determines reproducibility is complete restriction digestion. Many floral crops, including poinsettia, require optimization of extraction and digestion protocols for preparation of high-quality DNA requisite for reproducible polymorphic profiles (Barcaccia et al., 1999; Carr et al., 2003; J.H. Lyerly, unpublished data). Incomplete digestion can be detected in the AFLP pattern by loss of small AFLP fragments, along with gain of larger fragments (Life Technologies, Gaithersburg, Md.). Therefore, commonly occurring monomorphic fragments in the AFLP pattern, particularly those 100 bp and smaller, can serve as controls for complete restriction digest. Reliability and reproducibility of the AFLP technique is additionally insured by the stringent primer annealing conditions known as "touch-down PCR," which minimizes mispriming, and thus greatly reduces aberrant PCR products.

The repeatability of the AFLP banding patterns coupled with the validation of the polymorphisms by testing of multiple sources of various cultivars provides credibility to the AFLP fingerprints and the relationships concluded from them. Additional support of the data comes from the consensus of the different methods of analysis. The similar clustering in the dendrograms generated using different association coefficients and clustering methods verifies that the clusters are distinct (NTSypc 2.0, Exeter Software, Setauket, N.Y.). Likewise, the clustered groups in the principal coordinates analysis gives further support to the clusters.

Information in this study has shown that validation of polymorphisms is essential for the AFLP technique to be an effective and robust tool for identifying and differentiating poinsettia cultivars, as well as for determining breeding relationships. AFLP analysis of poinsettia and other floral crops provides valuable information that will facilitate the use of molecular methods for cultivar protection, support in breeding programs, and the potential to develop markers for desirable characteristics.

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